The **BLADE-ON-PETIOLE** genes are essential for abscission zone formation in *Arabidopsis*

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Sarah M. McKim, Grethe-Elisabeth Stenvik, Melinka A. Butenko, Wenche Kristiansen, Sung Ki Cho, Shelley R. Hepworth, Reidunn B. Aalen and George W. Haughn

The *Arabidopsis* **BLADE-ON-PETIOLE** 1 (**BOP1**) and **BOP2** genes encode redundant transcription factors that promote morphological asymmetry during leaf and floral development. Loss-of-function **bop1 bop2** mutants display a range of developmental defects, including a loss of floral organ abscission. Abscission occurs along specialised cell files, called abscission zones (**AZs**) that develop at the junction between the leaving organ and main plant body. We have characterized the **bop1 bop2** abscission phenotype to determine how **BOP1** and **BOP2** contribute to the known abscission developmental framework. Histological analysis and petal breakstrength measurements of **bop1 bop2** flowers show no differentiation of floral **AZs**. Furthermore, vestigial cauline leaf **AZs** are also undifferentiated in **bop1 bop2** mutants, suggesting that **BOP** proteins are essential to establish **AZ** cells in different tissues. In support of this hypothesis, **BOP1/BOP2** activity is required for both premature floral organ abscission and the ectopic abscission of cauline leaves promoted by the **INFLORESCENCE DEFICIENT IN ABSCISSION** (**IDA**) gene under the control of the constitutive CaMV 35S promoter. Expression of several abscission-related marker genes, including **IDA**, is relatively unperturbed in **bop1 bop2** mutants, indicating that these **AZ** genes respond to positional cues that are independent of **BOP1/BOP2** activity. We also show that **BOP1** and **BOP2** promote growth of nectary glands, which normally develop at the receptacle adjacent to developing **AZs**. Taken together, these data suggest that **BOP1/BOP2** activity is required for multiple cell differentiation events in the proximal regions of inflorescence lateral organs.

**KEY WORDS**: Abscission, **BLADE-ON-PETIOLE**, Flower development, **INFLORESCENCE DEFICIENT IN ABSCISSION**

INTRODUCTION

Plants alter their body plan in response to developmental and environmental cues by the addition of lateral organs, such as leaves and flowers. However, plant form is also shaped by the removal of these organs. Organs may be actively shed by the plant in a developmentally regulated process known as abscission (Lewis et al., 2006; Patterson, 2001; Roberts et al., 2002). Separation of organs from the plant body occurs at anatomically distinct cell files called abscission zones (**AZs**). Differentiated **AZ** cells are small, isodiametric and cytoplasmically dense compared with surrounding cells, and are responsive to signals promoting abscission. These signals induce a directed, enzymatic dissolution of the middle lamellae between **AZ** cell walls, resulting in a loss of adhesion between the organ and plant body (Addicott, 1982; Sexton and Roberts, 1982). Many enzymes have been suggested to be involved in the separation process, including cellulases, polygalacturonidasases (**PGs**) and expansins (Roberts et al., 2002). After the organ is shed, the cells exposed at the **AZ** on the plant body differentiate to form a protective surface (Patterson, 2001).

**Arabidopsis** petals, stamens and sepalas develop an abscission zone four to six cell layers thick, where the bases of the organs meet the receptacle (Bleecker and Patterson, 1997). Following fertilization, these floral organs senesce and abscise. Various genes are specifically expressed at floral organ **AZs** in *Arabidopsis*. For example, the promoter from an *Arabidopsis* abscission-related PG (**PGAZAT**) gene was able to drive floral organ **AZ**-specific expression of β-glucuronidase (**GUS**) during abscission (Gonzalez-Carranza et al., 2002). Furthermore, GUS constructs driven by the soybean (Glycine max) chitinase (**CHIT**) promoter or the bean (Phaseolus vulgaris) abscission cellulase (**BAC**) promoter are also upregulated in abscission zones during floral organ abscission (Bleecker and Patterson, 1997; Butenko et al., 2006; Chen and Bleecker, 1995; Patterson and Bleecker, 2004).

In several plant species, ethylene promotes abscission, whereas auxin inhibits it (Roberts et al., 2002). Although considerably delayed, floral organ abscission occurs in ethylene-insensitive mutants of *Arabidopsis*, suggesting that ethylene signalling is important for the timing of abscission but is not essential for it to occur (Patterson and Bleecker, 2004). Numerous loci are proposed to modulate floral organ abscission in *Arabidopsis*, including **HAESA** (Jinn et al., 2000), **AGAMOUS-LIKE** 15 (Fernandez et al., 2000), the **DELAYED ABSCISSION** loci (Patterson and Bleecker, 2004), genes for the actin-related proteins **ARP4** and **ARP7** (Kandasamy et al., 2005a), several **AUXIN RESPONSE FACTOR** genes (Ellis et al., 2005; Okushima et al., 2005), **INFLORESCENCE DEFICIENT IN ABSCISSION** (**IDA**) (Butenko et al., 2003), and **HAWAIIAN SKIRT** (Gonzalez-Carranza et al., 2007). Disruption of the expression of these genes results in delayed floral organ abscission, with the exception of **ida** plants, which retain floral organs indefinitely (Butenko et al., 2003). Floral organs of **ida** develop **AZs**; however, the middle lamellae only partially dissolve. **IDA** encodes a putative secreted peptide ligand suggested to act late in abscission to promote final middle lamellae dissolution (Butenko et al., 2003). Interestingly, ectopic and overexpression of **IDA** in 35S::**IDA** transgenic plants lead to precocious floral organ abscission and ectopic abscission at the...
vestigial AZs of pedicels and cauline leaves (Stenvik et al., 2006). Restricting IDA expression to the flower is likely to be crucial to prevent abscission at vestigial AZs.

Two recently identified Arabidopsis genes encoding redundant regulators of leaf and flower patterning, BLADE-ON-PETIOLE 1 (BOP1) and BOP2 (Ha et al., 2003; Ha et al., 2004; Ha et al., 2007; Hepworth et al., 2005; Norberg et al., 2005), were also shown to be required for floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). Loss-of-function bop1 bop2 mutants develop leafy projections from petioles and form floral bracts. In addition, flowers of bop1 bop2 develop two petaloid sepals instead of a single abaxial sepal. Furthermore, as in ida mutants, bop1 bop2 mutant floral organs fail to abscise. However, the molecular and anatomical basis of this defect has not been studied and it is unclear how the BOP1 and BOP2 genes function in the abscission process. BOP1 and BOP2 are part of the NPR1 (NON-EXPRESSOR OF PR1) protein family, which is characterised by a series of conserved cysteines and two protein–protein interaction domains. NPR1 is a positive regulator of systemic acquired resistance (SAR), a plant immune response induced following a local infection. During SAR, accumulation of salicylic acid causes a reductive shift in the cellular redox balance, prompting NPR1 to preferentially localise to the nucleus where it interacts with the TGACG sequence-specific binding transcription factors (TGAs) to activate the transcription of PATHOGENESIS RELATED (PR) genes (Dong, 2004). Several aspects of the BOP1/BOP2 signalling mechanism are conserved with NPR1, including localisation to both the cytoplasm and nucleus, and interaction with the TGA PERIANTHIA, which acts in the same genetic pathway to control perianth patterning in flowers (Hepworth et al., 2005). It is unknown whether BOP1/BOP2 activity is controlled by redox-control of nuclear localization and TGA interaction, as is the case for NPR1.

This study examines the contribution of BOP1 and BOP2 to abscission zone development. We show that BOP1 and BOP2 are required for abscission in both wild-type and 35S::IDA plants, as well as for all aspects of AZ-related anatomy in both functional and vestigial AZs. These data suggest that BOP1 and BOP2 promote formation of the specialized AZ anatomy necessary for abscission. Interestingly, the expression of abscission-related genes was relatively un perturbed in bop1 bop2 mutants, indicating that activation of AZ-specific gene expression is independent of AZ anatomy. In addition, our analysis determined that BOP1 and BOP2 promote nectary gland formation, which occurs at the receptacle adjacent to developing AZs. Thus, BOP1 and BOP2 regulate the differentiation of multiple cell types in proximal regions of inflorescence lateral organs.

MATERIALS AND METHODS
Plant material and growth conditions
Seedlings were germinated onto Arabidopsis minimal medium (Haughn and Somerville, 1986) and plants were grown in continuous light at 20°C as described (Dean et al., 2007). Wild type was the Columbia-0 (Col-0) ecotype, unless otherwise noted. Plants for 35S::IDA experiments were grown at 22°C for 8 hours dark/16 hours light and wild type was the C24 ecotype. T-DNA mutants bop1-3 (Col-0) and bop2-1 (Col-0) alleles have been described previously (Hepworth et al., 2005). T-DNA ida mutant (C24) and IDA::GUS (C24) lines have been described previously (Batenko et al., 2003). The 35S::IDA construct described by Stenvik et al. (Stenvik et al., 2006) was transformed into bop1 bop2 plants by floral dip (Clough and Bent, 1998). Analysis was conducted on the T2 generation. bop1 bop2 ida (Col-0×C24) triple mutants were generated by crossing. BOP1::GUS plants (Col-0) contain transcriptional fusions generated by fusing 4 kb upstream of the putative BOP1 start codon to the β-glucuronidase (GUS) reporter gene in the binary vector pBl101 (Jefferson et al., 1987). CHIT::GUS (Chen and Bleecker, 1995) and BAC::GUS (Koehler et al., 1996) marker lines have been introgressed into Col-0 and were provided by Dr Sara Patterson (University of Wisconsin, Madison, WA). CRABS CLAW (CRC)::GUS transgenic plants (Baum et al., 2001) and crc-1 mutants (Bowman and Smyth, 1999) were provided by Dr John Bowman (Monash University, Melbourne, Australia) and are both in the Landsberg erecta (Ler) background. Floral developmental stages were determined according to Smyth et al. (Smyth et al., 1990).

Petal breakstrength
Petal breakstrength is the force in gram equivalents required to remove a petal from the receptacle. Petal breakstrength was measured by the apparatus described by Lease et al. (Lease et al., 2006).

Microscopy and histology
For scanning electron microscopy of floral organ and vestigial AZs, sepals, petals and cauline leaves were removed prior to fixation. Following critical-point drying, tissues were mounted onto steel stubs, coated with gold palladium and observed using a Hitachi VP-4600 scanning electron microscope (Tokyo, Japan). For detection of GUS activity, tissue was fixed in 90% acetone, treated with heat and then rinsed with GUS buffer [100 mM NaH2PO4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide]. Samples were incubated with GUS buffer supplemented with 0.05% X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid cyclohexylamine) salt (Rose Scientific, Alberta, Canada) at 37°C for 2 hours for CRC::GUS and IDA::GUS plants, or overnight for BOP1::GUS, CHIT::GUS, GLUC::GUS plants. Tissues were cleared overnight in 70% ethanol, then cleared and mounted in chloral hydrate:water:glycerol (8:1:2). Tissues for sectioning were embedded in standard Spurr’s resin. BOP1::GUS and IDA::GUS samples were stained for GUS activity before embedding. For nectary and AZ analyses, sections were stained in Toluidine Blue.

Reverse transcriptase-mediated expression analyses
Total RNA from green rosette leaves, mature flowers and floral buds was isolated as described by Stenvik et al. (Stenvik et al., 2006). First-strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) was performed in a total volume of 20 μl using 1350 ng of total RNA as template, and incubated at 50°C for 60 minutes. Reverse transcription was omitted in negative controls. The open reading frame sequence of ACTIN2 (A13g18780) was used as a positive internal control. Primers used for ACTIN2 and IDA have been described by Stenvik et al. (Stenvik et al., 2006).

Real-time quantitative RT-PCR (qPCR)
Total RNA for qPCR was isolated from position 5-7 floral organ AZs. The reaction was performed on a LightCycler LC480 instrument (Roche) according to the manufacturer’s protocol. To ensure that primer combinations did not produce undesired PCR fragments or primer dimers, a SYBER-GREEN qPCR with melting-point analysis was performed using the LightCycler 480 SYBR Green Master Kit (Roche). Probe-based qPCR with these primers was performed using Universal Probe Library (UPL) hydrolysis probes (Roche), UPL probes 68 (IDA), 82 (HAESA) and 102 (ACTIN2), and the LightCycler 480 Probes Master Kit (Roche). All samples and reference controls were performed in two biological replicates and with two technical replicates each. Primers for amplification were: IDA 68 right, 5′-CCGGTACCATTGTCACACAC-3′; HAESA 82 left, 5′-GGACACTGGCAGATTAAACGAGG-3′; ACTIN2 102 right, 5′-CCGGTACCATTGTCACACAC-3′; and ACTIN2 102 left, 5′-CCGGTACCATTGTCACACAC-3′.

RESULTS
Loss of abscission in bop1 bop2 mutants is independent of senescence
Loss of floral organ abscission is a striking phenotype of bop1 bop2 plants (Fig. 1A). In wild type, sepals, petals and stamens normally abscise shortly after anthesis (flower opening). The convention used
BOP1 and BOP2 are necessary for AZ formation

The abscission defect of bop1 bop2 was further characterized using a petal breakstrength meter (Lease et al., 2006), which measures the amount of force required to remove a petal from the receptacle (Craker and Abeles, 1969). Owing to the progressive degradation of the middle lamellae in the AZ, breakstrength force decreased as the organs approach abscission (Fig. 1D). Dramatically, bop1 bop2 showed no decrease in breakstrength at any position, suggesting that cell-wall adhesion at the organ-receptacle boundary does not weaken in bop1 bop2 (Fig. 1D).

We used scanning electron microscopy (SEM) to examine the organ-receptacle boundary of bop1 bop2. Petals were removed at the receptacle if not yet abscised from wild-type and bop1 bop2 flowers at positions 2, 4, 6 and 12. The petal fracture surface of wild-type and bop1 bop2 position 2 flowers was composed of cells that ruptured upon petal removal (Fig. 2A,B, arrows). Owing to weakening of the middle lamellae, petal removal at position 4 (Fig. 2A) did not cause cell rupture but revealed a smooth fracture cavity that is typical of wild-type AZ cells (Bleecker and Patterson, 1997). At position 6, petals readily abscised to reveal a mature AZ characterised by spherical elongated cells (arrow). By position 12, the exposed plant body had differentiated into a protective surface. By contrast, the fracture surface of bop1 bop2 showed no evidence of AZ activation; cells ruptured during petal removal from all positions (Fig. 2B).

To determine more precisely the differences in cellular morphology between bop1 bop2 and wild type, longitudinal sections of flowers at anthesis were examined. Cytoplasmically dense AZ cells were visible in the stamen filiment-receptacle, petal-receptacle and sepal-receptacle boundaries of wild type (Fig. 2C-E, arrows), but such a layer never was observed in bop1 bop2 (Fig. 2G-I, arrows). Interestingly, the abaxial petalloid sepals of bop1 bop2 did not form an obvious junction with the floral receptacle – the cell layers were fused (Fig. 2I, arrow). In summary, analyses via petal breakstrength, SEM and light microscopy strongly suggest that cytologically distinct and active floral AZs do not form in bop1 bop2.

Vestigial AZs develop in Arabidopsis at branching points and at the base of pedicels and cauline leaves (Stenvik et al., 2006). Given the absence of floral AZs in bop1 bop2, we investigated whether bop1 bop2 also lacks vestigial AZs. To this end, SEM was performed on the vestigial AZs exposed after removal of cauline leaves. Fully expanded cauline leaves were removed from the stem at three maturation phases based on leaf colour: green, yellowing (50%) and yellow. The exposed fracture plane on wild type and bop1 bop2 stems following removal of green cauline leaves showed breakage of cells along the surface (Fig. 3A,D). Removal of yellowing cauline leaves from wild-type stems revealed enlarged rounded cells at the edges of the attachment point, indicative of an AZ (Fig. 3B), and when fully yellow, revealed rounded cells, although the vasculature cells remain broken (Fig. 3C). Corresponding surfaces of bop1 bop2 lacked any signs of these enlarged AZ cells (Fig. 3E,F). The bases of attached green cauline leaves were also examined. Abaxial surfaces of wild-type leaves at the stem showed a furrow of narrowed cells (Fig. 3G,H, arrows) flanked by stipules (Fig. 3H, arrowhead). This boundary furrow corresponded to the lower cleavage site formed when wild-type leaves are removed. Abaxial surfaces of bop1 bop2 cauline leaves lacked an obvious boundary furrow (Fig. 3J,K). Sections through expanded green wild-type cauline leaves revealed the narrowed cell layers of the leaf-stem boundary furrow (Fig. 3I, asterisk). Wild-type

### Table 1. Characterisation of floral organ abscission in wild type and bop1 bop2 ($n=70$)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Line</th>
<th>Sepals yellowing position ($\pm$ s.d.)</th>
<th>Floral organs withering position ($\pm$ s.d.)</th>
<th>Abscission position ($\pm$ s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous light</td>
<td>Col-0</td>
<td>2.56±0.79</td>
<td>5.40±0.50</td>
<td>6.55±1.09</td>
</tr>
<tr>
<td></td>
<td>bop1 bop2</td>
<td>2.59±0.82</td>
<td>6.76±0.83</td>
<td>na</td>
</tr>
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sections also showed darkened vestigial AZ cells at the adaxial leaf-stem boundary (Fig. 3I, arrow) and between the primary and axillary stem branching point (Fig. 3I, arrowhead). Wild-type vestigial AZs were readily apparent in serial sections through the sides of the leaf-stem boundary, but were less obvious through the vasculature (see Fig. S1 in supplementary material). However, neither cauline leaf nor branching point vestigial AZs were present in any position for expanded green \textit{bop1 bop2} cauline leaves; cells at the junction remained large and vacuolated (Fig. 3L). In summary, these data indicate that \textit{BOP1} and \textit{BOP2} are essential for vestigial AZ formation. Interestingly, stipules that flank the wild-type leaf fracture plane (Fig. 3A-C, arrow) and intact wild-type cauline leaves (Fig. 3H, arrowhead) were never observed flanking \textit{bop1 bop2} fracture planes (Fig. 3D-F) or intact leaves (Fig. 3K).

**BOP1** expression analyses

In situ hybridization has shown \textit{BOP1} and \textit{BOP2} mRNAs at the base of developing floral organs and in the AZ of mature flowers (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). We examined the expression pattern in more detail using a \textit{GUS} reporter gene under the control of the \textit{BOP1} promoter. \textit{BOP1::GUS} was expressed at the base of developing floral organs in stage 9/10 (Fig. 4A), stage 12 (Fig. 4B) and stage 14 (Fig. 4C) flowers, agreeing with previous results (Norberg et al., 2005). In addition, bases of recently shed organs had \textit{GUS} activity (Fig. 4D). \textit{BOP1::GUS} receptor expression was detected for a prolonged period following abscission (Fig. 4E). The adaxial regions of young pedicel-stem junctions showed \textit{GUS} activity (Fig. 4F) that spreads around the entire boundary in older pedicels (Fig. 4G). \textit{BOP1::GUS} expression exhibited similar patterns in \textit{bop1 bop2} as in wild type (data not shown), showing that \textit{BOP1} expression does not depend on functional BOP proteins. Interestingly, \textit{BOP1} was ectopically expressed in the \textit{bop1 bop2} pedicel where the ectopic bract develops (Fig. 4H, compare with 4G). In addition, expression of \textit{BOP1::GUS} was detected at the bases of cauline leaves (Fig. 4I), as previously reported (Norberg et al., 2005).

To further characterise \textit{BOP1} expression, \textit{BOP1::GUS} stained tissue was sectioned. Corresponding to in situ hybridisation results of \textit{BOP2} (Hepworth et al., 2005), \textit{BOP1::GUS} was expressed in early floral organ primordia (Fig. 4J). As the stamens become stalked, GUS activity was basally restricted (Fig. 4K, L), while the petal primordia, which develop later, displayed diffuse staining (Fig. 4L). The petal expression also became restricted to the petal base as petals grew out (data not shown). Mature flowers showed strong staining through the AZ (Fig. 4M) agreeing with \textit{BOP2} in situ hybridisation (Hepworth et al., 2005; Norberg et al., 2005). In summary, patterns of \textit{BOP1::GUS} expression correlate with a putative role in abscission zone development.
Suppression of *ida* and 35S::*IDA* phenotypes by *bop1 bop2*

The *ida* mutant shows a complete lack of abscission; however, unlike *bop1 bop2*, *ida* flowers differentiate floral organ AZs and have reduced petal breakstrength (Butenko et al., 2003). BOP1 and BOP2 are essential for anatomical differentiation of the AZ, suggesting that these roles are fulfilled prior to IDA function. To test this hypothesis, triple mutants were constructed and examined. The triple mutant *bop1 bop2 ida*, did not differentiate AZs or show decreases in petal breakstrength (see Fig. S2 in the supplementary material), demonstrating that *bop1 bop2* is epistatic to *ida*.

Absence of abscission in *bop1 bop2* is presumed to occur because of a lack of proper AZ anatomy. We tested whether overexpression of *IDA* by the CaMV 35S promoter in *bop1 bop2* plants can circumvent this requirement. Compared with wild type (C24) (Fig. 5A), precocious floral organ abscission was a striking phenotype of 35S::*IDA* plants (Fig. 5B), as previously described (Stenvik et al., 2006). These plants also exhibited activation of vestigial cauline AZs (Fig. 5F) and ectopic abscission at pedicels and branching points (Stenvik et al., 2006). Similar to *bop1 bop2* plants (Fig. 5C), 35S::*IDA* *bop1 bop2* plants showed no floral organ abscission (Fig. 5D), indicating that *bop1 bop2* is completely epistatic to 35S::*IDA*. Furthermore, ectopic abscission at vestigial AZs in pedicels, branching points (data not shown) and cauline leaves (Fig. 5F) was lost in 35S::*IDA bop1 bop2* plants, which resembled *bop1 bop2* (Fig. 5E). Both C24 and Col-0 backgrounds demonstrate these phenotypes when transformed with 35S::*IDA*, suggesting that the lack of 35S::*IDA* phenotype in 35S::*IDA bop1 bop2* is not due to the Col-0 ecotype. In addition, the 35S::*IDA* construct was not transcriptionally silenced in *bop1 bop2* (Fig. 5H).

Thus, earlier and ectopic expression of *IDA* is not sufficient to promote abscission in *bop1 bop2* because of an absence of proper AZ architecture.

Abscission-related gene expression in *bop1 bop2*

We were interested in determining whether BOP1 and BOP2 regulate expression of genes encoding putative signalling components, such as *IDA*, and/or enzymes normally transcribed in AZs. RT-PCR results indicated that *IDA*, *HAESA* and *HAWAIIAN SKIRT* had the same expression levels in *bop1 bop2* and wild-type flowers (results not shown). Whereas mutation in *HAWAIIAN SKIRT* leads to delayed abscission and fusion of sepal margins that precludes shedding (Gonzalez-Carranza et al., 2007), downregulation of *IDA* abscission and fusion of sepal margins that precludes shedding (Jinn et al., 2000; Butenko et al., 2003). The RT-PCR results were confirmed by quantitative PCR for *IDA::GUS* and *HAESA* expression was examined in Col-0 and *bop1 bop2* (Fig. 6A). Spatially, *IDA::GUS* expression was present at the base of the floral organs in *bop1 bop2*, similar to wild type (Fig. 6C,D).
CHIT::GUS maintained, albeit with lower intensity (Fig. 6H). Often bop1 bop2 structure in expression weakens (Fig. 6G). Despite the lack of anatomical AZ intensity, coincident with abscission at position 6, after which organ AZ cells starting at position 4 and increased to maximal not consistent.

Type (Butenko et al., 2006), BAC::GUS expression appeared early (before anthesis) and throughout the AZ by position 1 (Fig. 6E). BAC::GUS was expressed also in the proximal petal and stamen filaments. Although this temporal pattern was retained in bop1 bop2 (Fig. 6F), BAC::GUS expression was reduced in intensity, restricted to the bases of the petals and stamens and absent from single sepal AZ cells, as seen in the wild type (Fig. 6E, arrow).

Consistent with published results (Patterson and Bleecker, 2004; Butenko et al., 2006), CHIT::GUS was expressed in the floral organ AZ cells starting at position 4 and increased to maximal intensity, coincident with abscission at position 6, after which expression weakens (Fig. 6G). Despite the lack of anatomical AZ structure in bop1 bop2, temporal expression of CHIT::GUS was maintained, albeit with lower intensity (Fig. 6H). Often CHIT::GUS activity was detected earlier in bop1 bop2 but this was not consistent. CHIT::GUS expression also occurred in the proximal floral organs in the bop1 bop2 background (Fig. 6H). These data suggest that activation signals for BAC::GUS and CHIT::GUS are operating in bop1 bop2 regardless of a lack of AZ morphology. Interestingly, ida also shows normal temporal and spatial expression of both these reporters in AZ cells (Butenko et al., 2006), suggesting that signals activating their expression also function independently of IDA.

**BOP1 and BOP2 are necessary for nectary development**

Nectaries are secretory organs that develop from receptacle tissue after maturation of floral organs at stage 9 (Smyth et al., 1990), appearing as outgrowths at the base stamen filaments and connected to one another via a ring of nectary tissue that encircles cauline leaves do not abscise in bop1 bop2. (F) Cauline leaves of 35S::IDA show ectopic abscission. (G) 35S::IDA bop1 bop2 cauline leaves resemble bop1 bop2. (H) RT-PCR from cDNA derived from rosette leaves, buds and flowers from bop1 bop2 and 35S::IDA bop1 bop2. Upper panel shows RT-PCR with IDA primers. IDA is expressed in bop1 bop2 flowers only. 35S::IDA bop1 bop2 plants show expression in rosette leaves, buds and flowers. Lower panel, RT-PCR of ACT2-7 (ACT) as a positive control. Lane 1 is a genomic PCR control (g). l, leaves; b, buds; f, flowers.
Fig. 6. Expression of IDA::GUS, BAC::GUS and CHIT::GUS in wild type and bop1 bop2. Arabidopsis floral positions are indicated in the upper right of panels. (A) Wild-type (C24×Col-0) flowers at position 3 show very weak IDA::GUS activity in the base of floral organs that intensifies throughout position 4 and 5. (B) IDA::GUS expression in bop1 bop2 (Col-0×C24) is detected at position 4 and more strongly in position 5. (C,D) Dark-field microscopy of stained IDA::GUS flowers in wild type (C) and bop1 bop2 show staining throughout the proximal sepal. (E,F) BAC::GUS in wild type (Col-0) or bop1 bop2 at positions 1, 3-6 stained for GUS activity. (E) BAC::GUS plants show strong staining at the base of all floral organs, especially concentrated in the vasculature of position 1 and 3 flowers. Activity persists in position 4 and 5 flowers during abscission and is visible in single AZ cells (arrow). (F) BAC::GUS bop1 bop2 flowers at position 1 show weak staining at the bases of petals and stamens, and strengthens and enlarges by position 3. Very slight expression is detected on the adaxial side of sepals in position 4. (G,H) CHIT::GUS expression from flowers in wild type (Col-0) or bop1 bop2 at positions 3-7. (G) CHIT::GUS position 3 and 4 flowers demonstrate weak GUS staining, while positions 5 and 6 exhibit very strong AZ staining. (H) CHIT::GUS bop1 bop2 position 3 and 4 floral organs show weak GUS activity at their base. GUS activity is stronger in position 5. Staining is maintained in position 6 flowers extending further into the base of these floral organs. Arrowheads indicate expression. f, stamen filament; p, petal; s, sepal. Scale bars: 0.5 mm in A,B,E-H; 35 μm in C,D.

subtending the abaxial side. Medial nectaries are smaller and develop at the abaxial base of medial stamens. As the glands mature, they develop modified stomata designed for secretion and the cuticle becomes heavily reticulated (Baum et al., 2001). Nectaries showed strong BOP1 expression throughout nectary development (Fig. 4B,N).

During our AZ analyses, we noticed that bop1 bop2 flowers did not show obvious nectary glands; analysis of the F2 progeny of a bop1 bop2 × wild type cross demonstrated this phenotype cosegregated with other bop1 bop2 phenotypes (data not shown). Wild-type position 4 flowers with sepals and petals removed showed paired lateral nectary glands protruding at the base of the lateral stamens (Fig. 7A). By contrast, bases of bop1 bop2 lateral stamens lacked large nectary outgrowths (Fig. 7B). Wild-type lateral nectaries had characteristic deeply reticulated cuticle and associated secretory stomata (Fig. 7C), while in bop1 bop2, the area where lateral nectaries normally develop had slightly bulging areas of weak striation lacking secretory stomata (Fig. 7D).

From the onset of nectary development, two distinct nectary cell types exist: an outer epidermal layer and an inner starch granule-containing parenchymal tissue (Baum et al., 2001), as seen in transverse and longitudinal sections (Fig. 8E,G); a ridge of connecting nectary tissue also is present (Fig. 8E, arrow). The corresponding section of bop1 bop2 lacks distinct epidermal and parenchymal cells but shows slight bulges where the paired lateral glands would normally arise (Fig. 8F,H, arrows). Thus, although some residual cell division may occur, bop1 bop2 lacks differentiation of most nectary tissue characteristics.

Genetic interaction between CRABS CLAW and BOP1 and BOP2

CRABS CLAW (CRC) is a key gene regulating nectary development in Arabidopsis and encodes a putative zinc-finger transcription factor containing a YABBY domain (Bowman and Smyth, 1999; Siegfried et al., 1999). While bop1 bop2 flowers retain residual bulging reminiscent of nectary glands, crc mutants show a complete loss of nectary development (Bowman and Smyth, 1999). To determine whether loss of nectary growth observed in bop1 bop2 was due to misregulation of CRC expression, CRC::GUS expression was examined in bop1 bop2. Wild-type CRC::GUS expression was examined in Col-0×Ler background to compare with bop1 bop2 CRC::GUS plants generating by crossing. As described by Baum et al. (Baum et al., 2001), CRC::GUS is expressed in stage 7/8 flowers in the nectary gland anlagen (Fig. 7I); expression then expands throughout the connecting nectary tissue between the glands and is maintained after abscission of floral organs (Fig. 7JK). CRC::GUS is also expressed in stage 7/8 bop1 bop2 flowers (Fig. 7L), suggesting that BOP1 and BOP2 are
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Fig. 7. Nectary morphology and CRC::GUS activity in wild type (Col-0) and bop1 bop2. (A, B) Sepals and petals in Arabidopsis were removed from wild-type (A) and bop1 bop2 (B) position 4 flowers. Lateral nectary protrusions are obvious in wild type (arrow) but not in bop1 bop2. (C) Pair of lateral nectary glands with secretory stomata (arrow) in wild type. (D) Lateral nectary glands absent in bop1 bop2. (E-H) Toluidine Blue-stained sections from position 3 flowers. (E) Wild-type transverse section shows paired lateral nectary glands with distinct epidermal and starch-containing parenchymal regions, a ridge of connecting nectary tissue (arrow) and a medial nectary gland. (F) Corresponding bop1 bop2 cross-section shows two primordia (arrows) in the lateral nectary position. (G) A lateral nectary gland visible in longitudinal section of wild type. (H) Corresponding longitudinal section of bop1 bop2 shows a small outgrowth (arrow) in the lateral nectary position. (I-N) CRC::GUS plants stained for GUS activity. (I-K) CRC::GUS in wild type (Col-0×Ler). (I) Expression in the nectary anlagen of a stage 7/8 flower (arrow). (J) Strong nectary gland expression in a mature flower. (K) Position 7 flower with GUS staining nectaries and the ring of connecting nectary tissue. (L-N) CRC::GUS activity in bop1 bop2 (Col-0×Ler). (L) Expression in stage 7/8 flowers in the nectary anlagen (arrow). (M) Strong expression in the nectary gland region of a mature flower. (N) GUS staining in position 7 flowers shows strong staining in the lateral nectary gland region and weaker staining in the medial and connecting nectary tissue regions. In, lateral nectary; ls, lateral stamen; mn, medial nectary; p, petal; s, sepal. Scale bars: 500 μm in C, D; 70 μm in E-H, I-K, M, N; 35 μm in I, M.

not necessary for CRC expression in the nectary anlagen. As bop1 bop2 flowers mature, receptacle regions expressing CRC::GUS expand into lateral and medial nectary regions and in connecting areas although at reduced levels compared with wild type (Fig. 7M, N).

Like BOP1 and BOP2, CRC regulates other aspects of floral development. To determine whether these genes overlap in other functions, bop1 bop2 crc-1 triple mutants were constructed and examined. Nectary development was completely abolished in the triple mutants (data not shown) showing that the severe nectary phenotype of crc-1 is epistatic to that of bop1 bop2.

DISCUSSION

BOP1 and BOP2 act early to specify AZ anatomy

Abscission occurs in the AZ positioned at the junction between a lateral organ and the main plant body. The AZ is characterized by a unique anatomy and although it is thought to be essential for abscission, this has not been explicitly demonstrated in Arabidopsis as previously described mutants retain the development of an AZ. We show that two redundant NPR1-like homologues, BOP1 and BOP2, are expressed in the AZ and that lack of BOP1/2 proteins results in a complete absence of abscission. Moreover, the absolute loss of floral organ abscission in bop1 bop2 is uniquely correlated with an absence of cellular anatomy typical of the AZ, suggesting that the AZ anatomy is necessary for abscission and that BOP1 and BOP2 initiate differentiation of the AZ. However, we cannot rule out the possibility that BOP1 and BOP2 control other downstream events also necessary for abscission.

Several lines of evidence implicate that the specification by BOP1 and BOP2 of abscission zone cells as the earliest known step necessary for abscission. First, BOP1 and BOP2 genes are transcribed in early floral organ primordia, and resolve to a region corresponding to the future AZ prior to other known abscission-related genes. Second, appearance of AZ anatomy, which is absent in bop1 bop2, is the earliest identified event associated with AZ development. Third, the bop1 bop2 phenotype is epistatic to that of both ida and 35S::IDA, suggesting that the BOP1 and BOP2 act upstream of the only other gene known to be absolutely required for abscission.

BOP1 and BOP2 are expressed earlier than IDA, raising the possibility that BOP1 and BOP2 are positive regulators of IDA expression. However, we have shown that IDA is expressed similarly to wild type in bop1 bop2. It is possible that BOP proteins regulate IDA activity post-transcriptionally but given the requirement of BOP1 and BOP2 for AZ-specific anatomy, we favor a model where BOP1/2 function early to specify the AZ cell type and IDA acts relatively independently to finalize the cell separation process. Significantly, as is the case for IDA (Butenko et al., 2006), BOP1 and BOP2 are not required for the correct temporal transcription of known abscission-related genes tested here, including one encoding a cell wall hydrolytic enzyme.

A model of the known essential players in abscission is presented in Fig. 8. We propose that BOP1 and BOP2 act early to specify AZ-unique anatomy. The characteristics of this anatomy that make it crucial for abscission remain to be identified but could include cell shape and/or cell wall structure amenable to middle lamellae
digestion. Upstream factors that regulate initiation of abscission act through both ethylene-dependent and ethylene-independent pathways that converge to activate the expression of abscission-related genes, including middle lamella degrading enzymes. These enzymes are expressed in the AZ, presumably with specific spatial and temporal profiles, to progressively weaken the middle lamellae. This expression is driven independently from BOP1/2-mediated differentiation of the AZ. As yet, none of these enzymes has individually been shown as essential for abscission. Finally, IDA is necessary for abscission and is expressed in the AZ just prior to abscission in response to an unknown signal. Given that partial enzymatic dissolution of the middle lamellae occurs in ida mutants, IDA must act downstream from the initiation of abscission. Expression of IDA alone is insufficient for abscission as premature abscission of 35S::IDA plants occurs only at flower positions with differentiated AZ (Stenvik et al., 2006), and is dependent on BOP1 and BOP2-mediated differentiation of the AZ. The specific role of IDA and its putative receptor are unknown but must be involved in the final essential steps of the cell separation process.

**BOP1 and BOP2 also specify vestigial AZ anatomy**

Although *Arabidopsis* leaves do not abscise, ectopic abscission induced by 35S::IDA has suggested that vestigial AZs develop at the bases of cauline leaves, branching points and at the base of pedicels (Stenvik et al., 2006). Several lines of evidence presented here strongly support this hypothesis. First, cauline leaf AZs have characteristic AZ anatomy and a boundary furrow that demonstrates progressive degradation of middle lamellae with age similar to floral AZs. Second, the characteristic anatomy, boundary furrow and 35S::IDA-induced abscission at these putative vestigial AZs are dependent on BOP activity. Finally, vestigial AZs develop on the adaxial side of the leaf base, corresponding well to *BOP1* and *BOP2* expression, as shown here and in other studies (Ha et al., 2004; Norberg et al., 2005). Taken together, these data suggest that vestigial AZs do develop and that their anatomy is regulated by *BOP1* and *BOP2*.

**Nectary formation and the role of BOP1 and BOP2**

Nectaries are not entirely absent in *bop1 bop2*, but rather are greatly reduced in size and do not differentiate key nectary features such as parenchymal and secretory tissue, and modified stoma. Our analysis suggests that *bop1 bop2* mutants retain *CRC*:GUS activity in both the nectary anlagen and the bulges that later develop, indicating that the lack of nectary outgrowth is not due to a loss in *CRC* expression. Similar to *CRC*, *BOP2* is expressed very early in nectary development and may be controlling other downstream elements in conjunction with *CRC*.

Formation of the third whorl, although not the presence of stamens in this whorl, is essential for nectary formation (Baum et al., 2001). Third whorls in *bop1 bop2* often develop an extra medial stamen between the other two medial stamens and the adjacent petal on the abaxial side (Hepworth et al., 2005). Given this additional growth, the nectary phenotype in *bop1 bop2* may be a secondary effect of ectopic growth around the stamen attachment areas.

**The role of BOP1 and BOP2 in plant development**

Previous research on *BOP1* and *BOP2* has suggested a role in defining the identity of the proximal regions of lateral organs (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). The receptacle may be thought of as a proximal feature of a flower, just as the petiole is a proximal area of a leaf. Therefore, the absence of AZs and reduction in nectaries in *bop1 bop2* suggests that *BOP1* and *BOP2* regulate multiple differentiation events in the proximal flower. Prior work has demonstrated that *BOP1* and *BOP2* may repress class I Knox gene expression in the shoot (Ha et al., 2003; Ha et al., 2007). Class I Knox genes are important to maintain and establish shoot meristem identity and are normally downregulated in incipient lateral organ primordia (Scofield and Murray, 2006). Temporal and/or spatial misregulation of class I Knox genes in developing AZs may contribute to defects in AZ differentiation.

Although AZs may be thought of as defining the organ-plant body junction, cauline leaves in *bop1 bop2* do not display gross malformations where they meet the stem. Furthermore, expression of floral organ AZ markers, such as *IDA* and *HAESA*, persists in *bop1 bop2*, suggesting the positional information is intact. Thus, *bop1 bop2* plants lack some but not all of the features of the organ-plant body junction, suggesting that other factors are responsible for overall boundary patterning, such as *CUP-SHAPED COTYLEDONS* (Aida and Tasaka, 2006), while the *BOP1* and *BOP2* genes are later effectors of specific aspects of the organ-plant body interface.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1537/DC1
References